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14. ABSTRACT

The molecular mechanisms and genetic pathways involved in the pathogenesis of myeloproliferative disease (MPDs) are not well understood. Telomere maintenance and the cellular responses to telomere dysfunction have been proposed to play crucial roles in the processes of genomic stability, aging, organ homeostasis and tumorigenesis. Our recent observation linking telomere dysfunction to an MPF like phenotype with thrombocytosis in the telomerase mutant mouse model provides an unique genetic platform to explore the molecular mechanisms by which telomere dysfunction contributes to the pathogenesis of MPD and essential thrombocytosis (ET). We hypothesize that mice engineered to have critically short telomeres will develop genomic instability which predispose these mice to the development of MPD/ET. In vitro and in vivo analyses of the hematopoietic compartment from these mutant mouse cohorts as a function of age and generation in the last 2 years have elucidated some of the genetic pathways that are involved in the pathogenesis of MPDs/ET.

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Table of Contents

Introduction:

The molecular mechanisms and genetic pathways involved in the pathogenesis of myeloproliferative disease (MPDs) are not well understood. Telomere maintenance and the cellular responses to telomere dysfunction have been proposed to play crucial roles in the processes of genomic stability, aging, organ homeostasis and tumorigenesis. Our recent observation linking telomere dysfunction to an MPF like phenotype with thrombocytosis in the telomerase mutant mouse model provides an unique genetic platform to explore the molecular mechanisms by which telomere dysfunction contributes to the pathogenesis of MPD and essential thrombocytosis (ET). We hypothesize that mice engineered to have critically short telomeres will develop genomic instability which predispose these mice to the development of MPD/ET. In *vitro* and *in vivo* analyses of the hematopoietic compartment from these mutant mouse cohorts as a function of age and generation in the last 2 years have elucidated some of the genetic pathways that are involved in the pathogenesis of MPDs/ET.

Body:

Task 1: Identification of the cellular basis of MPD in aged telomere dysfunctional mice.

Telomere analyses of bone marrow derived from telomere dysfunction mice with MPD (Maximum 25 mice) (Months 1-12). We will utilize modified telomere fluorescent probe hybridization and FACS (FLOW-FISH) on sorted bone marrow cells from the different mutant mice and quantitative fluorescent telomere probe hybridization to metaphase chromosomes derived from sorted bone marrow culture of various mutant mice to determine the telomere lengths.

We have quantified the telomere signals from metaphase spread of sorted bone marrow cells from the various cohorts using telomere FISH analyses combined with quantitative imaging software TFL-Telo and showed that the all the sorted bone marrow population has a more severe telomere shortening from aged telomere dysfunctional mice

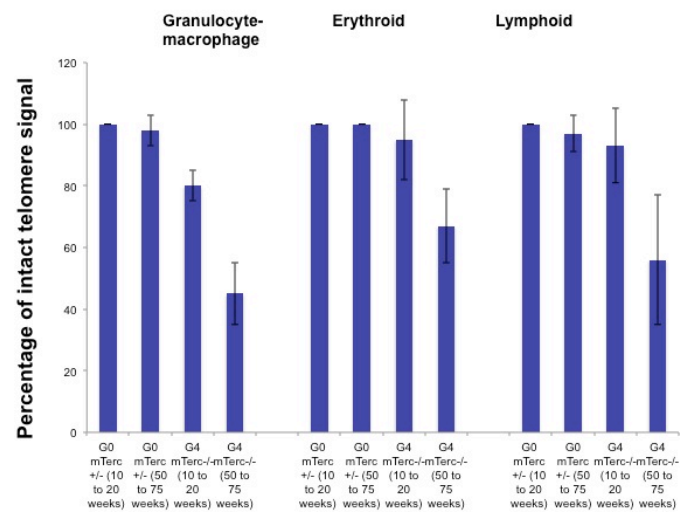On the average the granulocyte-macrophage, erythroid and lymphoid sorted

Figure1. Telomere dysfunctional bone marrow cells have critically shortened telomeres. FLOW FISH analyses were performed to the respective sorted cell population from the various mTerc mutant mouse cohorts and their signal is normalized to the G0 mTerc 10-20 weeks old mice. Each cohort has at least 5 mice.

bone marrow population from the aged G4 mTerc mutant mice with telomere dysfunction have a 50% (p value of <0.05) reduction in telomere signal as compared to the comparable aged G0 mTerc mutant mice (Figure 1). These studies were performed with on an average of 5 mice from each cohort.

Hematopoietic stem cell (HSC) analysis of aged telomere dysfunctional mice. (Maximum 25 mice) (Months1-12). We will ascertain the combined impact of age and telomere erosion on the population of HSC, and other committed progenitors including CMP (common myeloid progenitor), GMP (granulocyte monocyte progenitor), and MEP (megakaryocyte erythroid progenitor) populations by determining the percentage of these cells in the bone marrow of the different cohorts of telomerase mutant mice using high speed five color flow cytometric analysis.

We have observed a significant 30% ($p<0.05$) increase in the proportion of granulocyte-macrophage progenitors (GMPs) at the expense of CMP and MEP populations in the aged G4 mTerc mutant telomere dysfunctional mice as compared to the G0 mTerc +/- 10 to 20 week mouse controls, which matches the dramatic increase in immature developing granulocytes in the bone marrow and spleen (Figure 2).

Further, we have isolated RNA from each of the four isolated cell populations (HSC, CMP, GMP, and MEP) to help determine whether master transcription regulator pathways of hematopoietic development are perturbed in telomere dysfunctional mice. Microarray analyses following whole

Figure 2 Telomere dysfunctional bone marrow cells have increased GMP population and decreased CMP and MEP population. FLOW analyses were performed in the various mTerc mutant mouse cohorts and the various subpopulation of cells is normalized to that of the G0 mTerc 10-20 weeks old mice. N= number of mice in the cohort

transcriptome amplification of purified RNA from the GMP population has been performed and has revealed an intriguing increase in RNA levels of several indicators of active IL-6 receptor and FLT3 signaling, as well as interferon responses. This demonstrated that telomere dysfunction either directly or indirectly induces altered gene expression patterns that promote an abundance of GMPs, leading to an expansion of this lineage in the bone marrow and periphery.
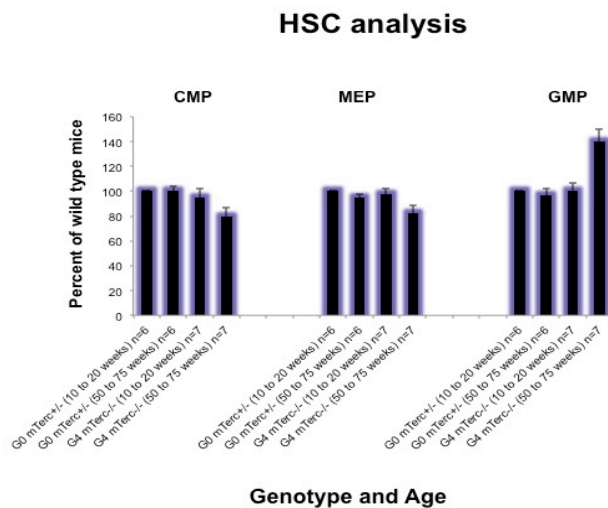
Growth factor independent clonogenic growth of bone marrow cells from aged telomere dysfunctional mice (Maximum 15 mice) (Months 1-12). We will test the ability of
progenitor cells from aged telomere dysfunctional mice to grow in the absence of exogenously added growth factors using methycellulose-containing media without exogenous cytokines and scoring for colony forming ability.

Colony forming assays have been performed on the G4 telomere dysfunctional mice and relevant age-matched controls.
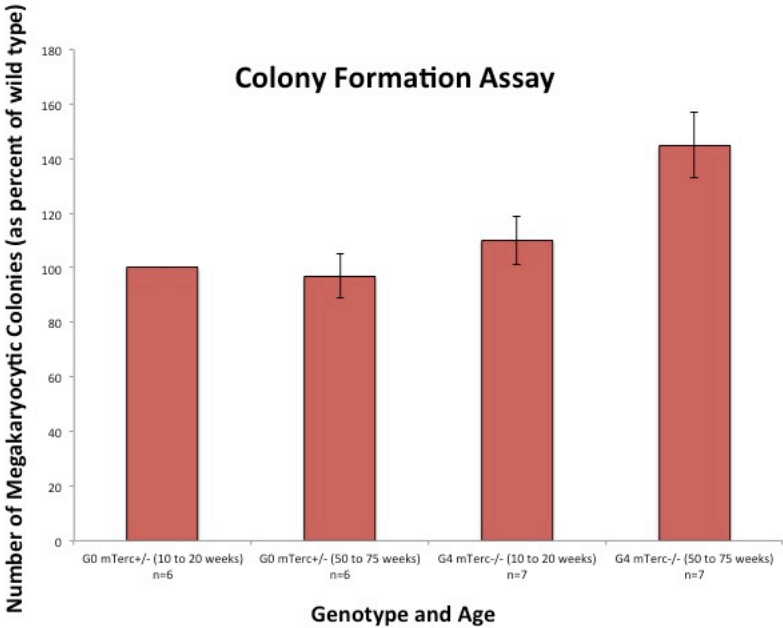
There is an approximately 35 percent increased in megakaryocytic colony formation from cells derived from the G4 telomere dysfunctional mice as compared to the G0 mTerc controls (Figure 3). This is consistent with the data above in which we demonstrated that there is an increased in granulocyte-macrophage progenitors (GMPs) in the G4 telomere dysfunctional mice. There were no significant differences in the number of colonies from the other progenitors.

Figure 3. Increased megakaryocytic colonies from bone marrow of G4 mTerc mutant mice with telomere dysfunction. Equal number of bone marrow cells from the mice of the various indicated cohorts are plated on to methylcellulose-containing media and the various colonies are scored after 7 days. The number of colonies is then normalized to the G0 mTerc 10-20 weeks controls N= number of mice in the cohort.

In vitro characterization of HSC progenitor populations from aged telomere dysfunctional mice (Maximum 25 mice) (Months 1-12). We will determine the effect of telomere dysfunction in the bone marrow cell populations of aged telomerase deficient mice using cell culture-based colony

CBC of telomere mutant mice

	HCT %	Granulocytes (K/ml)	Plt (K/ml)
G0 mTerc+/- (10 to 20 weeks) n=6	52 +/- 7	6.4 +/- 3	2345+/- 323
G0 mTerc+/- (50 to 75 weeks) n=6	55 +/- 9	5.4 +/- 2.9	1979 +/-345
G4 mTerc-/- (10 to 20 weeks) n=7	42 +/-8	13.8 +/- 4.1	2598 +/- 429
G4 mTerc-/- (50 to 75 weeks) n=7	23 +/- 10	18.3 +/- 5	5460 +/-358

Table 1. CBC of from the peripheral blood of the various cohorts. At least 5 mice were measured from each cohort.

forming assays, long-term colony initiating cell assays, and serial replating assays.

We further confirmed that there is a substantial increase in the proportion of granulocyte-macrophage progenitors (GMPs) at the expense of CMP and MEP populations using the long term colony initiating cell assays and serial replating assays. Again, an average of 5 to 7 mice were used from the control and the experimental G4 mTerc mutant telomere dysfunctional mice.

Cytokine production by telomere dysfunctional bone marrow derived cells (Maximum 25 mice) (Months1-12). We will test whether the cytokine production is intrinsic to the MPD cells in aged telomere dysfunctional mice using the Luminex xMAP technology (bead-bound sandwich ELISA; Luminex, Austin, TX), allowing simultaneous detection of up to 20 cytokines from each sample

As shown previously, we have confirmed that telomere dysfunction is associated peripheral blood red cell anemia, neutrophilia, and thrombocythemia as seen in the aged G4 mTerc mutant telomere dysfunctional mice as compared to the G0 mTerc controls (Table 1).

Figure 4. IL6, IL1B and TNF alpha concentration from the serum of mice from the various indicated mouse cohorts. Each point represents the value from a single mouse. At least 6 mice were measured from each cohort.

These alterations were accompanied by substantial quantitative increases in plasma erythropoietin and several inflammatory cytokines, notably IL-6, IL1B and TNF alpha (Figure 4) .

These alterations in peripheral blood were aslo accompanied by histological changes elsewhere in the hematopoietic system. In the spleen we noted extensive extramedullary hematopoiesis and splenomegaly, and subsequent loss of lymphoid follicles and replacement by granulocytic lineages. Finally, in the bone marrow, we noted hypercellular bone marrows primarily made up of developing granulocytic lineages with a corresponding loss of developing erythroid and lymphoid lineages.

All the above in vitro analyses are consistent with this histological phenotype.

In vivo characteristics of Bone Marrow Compartment of aged telomere dysfunctional mice with MPD

Transplantability of the MPD phenotype from bone marrow cells of telomerase null mice with telomere dysfunction. (Maximum 40mice) (Months 6-18). To test whether the MPD phenotype of the telomerase null mice with telomere dysfunction can be transplanted into recipient mice, we will perform adaptive transfer experiments.

Several rounds of transplantation of control and aging telomere dysfunctional whole bone marrow into isogenic recipients have been performed which further demonstrated the dramatic increase in GMP and decrease in MEP progenitor populations as noted by FACS sorting of the recipient mice of the marrow from the aging G4 mTerc mutant telomere dysfunctional mice.

The bone marrows and spleens from

recipient mice of the experimental G4 mTerc mutant telomere dysfunctional mice   are nearly completely populated by granulocytic lineages derived from the donors as compared to the normal marrows and spleens of recipient mice from the control mice.

The recipient mice from marrow of the experimental G4 mTerc mutant telomere dysfunctional mice succumbed quickly (Table 2, $P<0.05$ for comparison between G0 wild type donors and G4 mice with dysfunctional telomere donors) and likely due to anemia and thrombocytopenia since fairly few MEP between G4 mTerc 50 to 70 week bone marrow to G0 mTerc+/- 10 to 20 week controls) progenitors reside in the telomere dysfunctional telomere bone marrow.

alive after 8 weeks of transplantation of mTerc mutant mice into wild type mice recipient	
G0 mTerc+/- (10 to 20 weeks) n=20	20
G0 mTerc+/- (50 to 75 weeks) n=20	20
G4 mTerc-/- (10 to 20 weeks) n=20	16
G4 mTerc-/- (50 to 75 weeks) n=20	7

Table 2. Number of recipient mice alive after 8 weeks of transplantation from bone marrow of the indicated mutant mouse cohorts. Bone marrow from 5 donor mice from each cohort was used to transplant into 20 recipient wild type mice

wild type mice bone marrow transplantation into irradiated mTerc mutant mice	HCT %	Granulocytes (K/ml)	Plt (K/ml)
G0 mTerc+/- (10 to 20 weeks) n=10	56 +/- 5	5.6 +/- 2.7	2134 +/- 34
G0 mTerc+/- (50 to 75 weeks) n=10	54 +/- 7	7.9 +/- 4.8	1946 +/-114
G4 mTerc-/- (10 to 20 weeks) n=10	57 +/8	7.9 +/- 8.3	1897 +/- 230
G4 mTerc-/- (50 to 75 weeks) n=10	46 +/- 8	12.3 +/- 3.9	2120 +/-502

Rescue of the MPD phenotype in telomerase null mice with telomere dysfunction with HSCs

Table 3. CBC of the recipient mutant mice of the various cohorts 8 weeks after transplantation with wild type bone marrow cells. N= number of mice in the cohort.

from wild type mice. (Maximum 40 mice) (Months 6-18). Either dysfunctional HSCsor/and altered bone marrow stromal environment may cause the MPD phenotype seen in the telomerase null mice with telomere dysfunction. To determine the contribution of the bone marrow stromal environment on the MPD phenotype, we will determine whether HSC from wild type mice can rescue the MPD in the telomerase null mice.

To directly test whether the role of HSCs and stroma, we have performed whole bone marrow transplantation of wildtype bone marrow (unfractionated) into sublethally irradiated G4 *Terc* telomere dysfunctional mutant mice. We showed that wildtype cells can largely develop normally in a *Terc* mutant telomere dysfunctional background. In the peripheral blood, wildtype B and T cell production was largely restored the telomere dysfunctional recipient mice (Table 3). This indicates that the stromal environment of *Terc* telomere dysfunctional mutant mice is conducive to largely normal blood cell production.

In conclusion, we demonstrated that the altered bone marrow environment in the G4 mTerc mutant telomere mice is not likely to substantially contribute to the MPD phenotype. Instead, the experimental evidence points to defective/altered telomere dysfunctional HSCs

Conventional cytogenetics and BCR-ABL analysis. (Months 6-18). To differentiate the MPD phenotype in aged telomere dysfunctional mice, and to correlate the phenotype to human diseases associated with MPD, we will determine whether the MPD with neutrophilia is a form of chronic myeloid leukemia related to BCR-ABL rearrangement. Thus, we will perform fluorescent in situ hybridization (FISH) and Northern analyses to determine if telomere dysfunctional mice harbor BCR-ABL rearrangements in bone marrow precursor and maturing granulocyte lineages.

FISH and Northern analyses on the hematopoietic bone marrow precursor cells derived from the aged telomere dysfunctional mice showed that these cells do not harbor the BCR-ABL rearrangements. This demonstrated that the MPD phenotype seen in our mutant mice is not caused by the BCR-ABL rearrangement and thus the mechanism for the emergence of this phenotype might be novel.

Spectral karyotype (SKY) analysis of MPD metaphases. (Months 12-24). Given the relationship between genomic instability and progression to CML blast phase, as well as the known genetic rearrangements associated with hematological malignancies, we will perform SKY analyses to comprehensive assess the cytogenetic profile of the bone marrow cells from aged telomere dysfunctional mice

SKY analyses of the MPD metaphases demonstrated that these cells do not harbor any gross clonal chromosomal rearrangements. However, this does not preclude the existence of focal rearrangements that cannot be detected by SKY.

Array-based whole genome analyses. (Months 18-24). Because genomic instability and bridge-fusion-breakage cycles are often associated with regional amplifications and

deletions of genomic loci, we will employ oligonucleotide array comparative genomic hybridization (CGH) to determine MPD-specific genetic loci in telomere dysfunctional mice.

We have isolated GMP populations from wildtype and telomere dysfunctional MPD-like affected bone marrow (5 mice each). CGH base analysis did not revealed any gross genetic aberrations

Kinase cascade analysis. (Months 18-24). A large body of evidence demonstrates the importance of tyrosine kinase mutations to the development of myeloproliferative disorders, particularly CML. Clues regarding the relative importance of various kinase pathways can be gleaned from examining the activation state of various molecular players in the kinase cascades. Thus, as a corollary analysis to the DNA- and RNA- based analyses described above, we will undertake an examination of phosphorylation events that occur in mice with MPD using the Luminex xMAP bead-bound sandwich immunoassay (Bio-Rad Laboratories, Hercules, CA).

Analysis of signaling cascades active in telomere dysfunctional MPD-like bone marrow as compare to the normal controls did not revealed any differential phosphorylation events in the signal transduction cascades using the *Luminex xMAP bead-bound sandwich immunoassay (Bio-Rad Laboratories, Hercules, CA)*

Task 3: The impact of ionizing radiation, chronic replicative stress, and other genetic alterations on the progression of the MPD phenotype in aged telomere dysfunctional mice.

In vivo chronic stress on the hematopoietic system of the telomerase null mice with telomere dysfunction with 5-FU. (Maximum 40 mice) (Months 12-24) We hypothesize that chronic stress on the hematopoietic compartment to expand in the aged telomere dysfunctional mice will further fuel the genomic instability generated from progressive
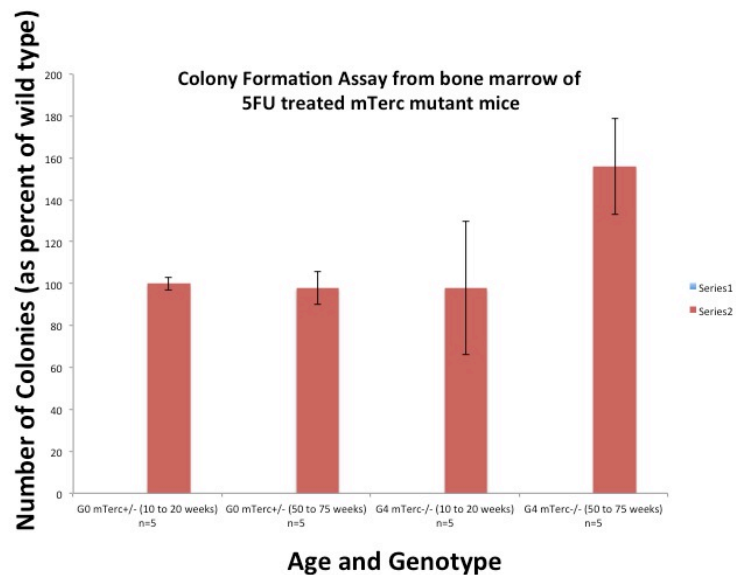

Figure 5 5FU treated telomere dysfunction bone marrow has increased megakaryocytic colonies. Equal number of bone marrow cells from the 5FU treated mice of the various indicated cohorts are plated on to methylcellulose-containing media and the various colonies are scored after 7 days. The number of colonies is then normalized to the G0 mTerc controls. N= number of mice in the cohort.

telomere dysfunction in the stem/progenitor cells and cause either its depletion or transformation.

We treated the cohorts of the G4 mTerc mutant mice with telomere dysfunction and normal G0 controls with 5FU at (50mg/kg body weight) once every 3 weeks for a total of 5 cycles and subsequently analyzed their bone marrows. As before, we confirmed that there is a substantial increase in the proportion of granulocyte-macrophage progenitors (GMPs) as seen from our previous data. This is further confirmed by the colony formation assay in which there is greater number of megakaryocytic colonies from bone marrow of the 5FU treated telomere dysfunctional mice as compared to the G0 mTerc controls (Figure 5). The chronic stress induced from 5-FU did not seem to further exacerbate the phenotype as further confirmed by the CBC of these 5FU treated mice from the various mouse cohorts (Table 4).

5FU treated mTerc mutant mice

	HCT %	Granulocytes (K/ml)	Plt (K/ml)
G0 mTerc+/- (10 to 20 weeks) n=10	42 +/- 8	8 +/- 3	1459+/-1209
G0 mTerc+/- (50 to 75 weeks) n=10	49 +/- 12	7.4 +/- 2.1	1896 +/-114
G4 mTerc-/- (10 to 20 weeks) n=10	51 +/9	11.3 +/- 2.3	3456+/- 830
G4 mTerc-/- (50 to 75 weeks) n=10	21 +/- 4	19.3 +/- 9	6750 +/-1002

Table 4. CBC of the 5FU treated mice from the various cohorts. CBC was taken 6 weeks after the last cycle of 5FU treatment N= number of mice in the cohort

In vivo genotoxic stress on the hematopoietic compartment of the telomerase null mice with telomere dysfunction with sublethal dose of ionizing radiation. (Maximum 40 mice) (Months 12-24). Our previous studies have shown that telomerase null mice are hypersensitive to ionizing radiation and that telomerase null cells with telomere dysfunction are impaired in their ability to repair double strand DNA breaks. Exposure to ionizing radiation will generate greater genomic instability leading to greater mutational rate and stem/progenitor cell depletion. A transformed clone might emerge from the natural selection process. To test this hypothesis, we will expose various cohorts of telomerase mutant mice to 3Gy of sub-lethal ionizing radiation and then monitor them for development of worsening phenotypes.

We determined that after a sub-lethal dose of radiation (3 Gy), there is no worsening of the phenotype as measured by CBC (Table 5) 10 weeks after radiation treatment. At the time of natural death from the radiation treated cohorts, histological analyses of the bone marrow also did not reveal any evidence of leukemia. As with the untreated mice, only MPD is seen in the radiation treated mTerc mutant mice with telomere dysfunction.

Cooperativity of mutations in transcriptional factors and activated tyrosine kinases known to be relevant in AML on the progression of disease in the telomerase null mice. (Maximum 100 mice) (Months 12-24). To investigate whether there is cooperativity between telomere dysfunction and known mutant proteins involved in the process of MPD or leukemia, we will retrovirally introduce these mutant proteins into the bone marrow cells of the aged telomere dysfunctional mice and transplant them into recipient mice. These recipient mice will then be closely monitored for worsening of the MPD phenotype.

3Gy sub-lethal radiation

	HCT %	Granulocytes (K/ml)	Plt (K/ml)
G0 mTerc+/- (10 to 20 weeks) n=10	49 +/- 6	5.8 +/- 6	1346 +/- 720
G0 mTerc+/- (50 to 75 weeks) n=10	56 +/- 9	6.8 +/- 2.1	1234 +/-649
G4 mTerc-/- (10 to 20 weeks) n=10	48 +/7	8.9 +/- 2.3	2345 +/- 546
G4 mTerc-/- (50 to 75 weeks) n=10	19 +/- 8	22.3 +/- 6.5	6789 +/-789

Table 5. CBC of the irradiation treated mice from the various cohorts. CBC was taken 10 weeks after the irradiation treatment or before natural death. N= number of mice in the cohort

MSCV-based retroviruses expressing BCR-ABL and FLT3-ITD, PML-RAR and NUP98-Hoxa9 have been transduced into telomere dysfunctional bone marrow and wild type controls. Transduction of wildtype bone marrow was quite effective and these infected bone marrow were transferred to recipient mice. Unfortunately, none of these four mutants cooperated with telomere dysfunctional bone marrow to progression to more severe form of MPD or more sever form of leukemia when compared to the transduced normal bone marrows.

Key Research Accomplishments:
Over the past 2 years we have solidified our initial observations, which include an age- and telomere dysfunction associated peripheral blood red cell anemia, neutrophilia, and thrombocythemia. These alterations were accompanied by substantial increases in plasma erythropoietin and several inflammatory cytokines, notably IL-6 TNF alpha and IL1B. We have also shown that many of the phenotypes seen are transplantable; this point is important, as this contradicts previous literature in this field. We demonstrated that these effects are due to telomere dysfunction, rather than *Terc* gene deficiency *per se*. We have now shown that there are not known changes in down stream signal transduction pathways in these telomere dysfunctional bone marrow. Additional stress by chronic treatment with 5FU or sub-lethal radiation does not further exacerbate this phenotype. Lastly, we showed that BCR-ABL and FLT3-ITD, PML-RAR and NUP98-

Hoxa9 do not cooperate with telomere dysfunction to progress this MPD phenotype seen in the telomere dysfunctional mice

Reportable Outcomes: We have confirmed that telomere dysfunctional mice have an telomere dysfunction associated peripheral blood red cell anemia, neutrophilia, and thrombocythemia. These alterations were accompanied by substantial increases in plasma erythropoietin and several inflammatory cytokines, notably IL-6, TNF alpha and IL1B. We have also shown that many of the phenotypes seen are transplantable. We demonstrated that these effects are due to telomere dysfunction, rather than *Terc* gene deficiency *per se*. We also have shown that there are not known changes in down stream signal transduction pathways in these telomere dysfunctional bone marrow. Additional stress by chronic treatment with 5FU or radiation does not further exacerbate this phenotype. Lastly, we showed that BCR-ABL and FLT3-ITD, PML-RAR and NUP98-Hoxa9 do not cooperate with telomere dysfunction to progress this MPD phenotype seen in the telomere dysfunctional mice.

Conclusions: We have confirmed that the MPD that develops in Terc mutant mice is indeed cell intrinsic. Importantly, these phenotypes are due to telomere dysfunction, not deficiency of Terc *per se.* Telomere dysfunctional mice were largely competent to allow engraftment of wildtype bone marrow, indicating that the stromal environment is largely conducive to normal bone marrow growth.

References: none